REMARKS/ARGUMENTS

Claims 14-38 were pending in the present application. By virtue of this response, claims 15 and 17-21 have been cancelled, claims 14, 16, 22, 26, 30 and 34 have been amended. Accordingly, claims 14, 16, 22-38 are currently under consideration. Support for the present claim amendments can be found throughout the specification and claims as originally filed, for example, at page 4, lines 15-30; page 6, line 28 to page 7, line 15. Amendment and cancellation of certain claims is not to be construed as a dedication to the public of any of the subject matter of the claims as previously presented. No new matter has been added.

The Sequence Listing

The sequence listing has been corrected as required in the present Office Action.

The undersigned hereby states that the paper copy of the substitute Sequence Listing and the computer readable form copy of the substitute Sequence Listing, submitted in accordance with 37 C.F.R. § 1.825(a) and (b), respectively, are the same and contain no new matter. Accordingly, entry of the substitute Sequence Listing into the above-captioned application is respectfully requested.

The Pending Claims

The Applicant respectfully asserts that original claim 14 claimed a pharmaceutical PTH antagonist composition wherein the PTH antagonist contained particular characteristics, wherein the N-terminal amino acid residue of the PTH antagonist started at any position spanning from position 2 through position 34 of SEQ ID NO:1 (PTH_{1-84}); and the C-terminal amino acid residue of the PTH antagonist ended at position 84 of SEQ ID NO:1 (PTH_{1-84}). As amended, claim 14 claims a PTH antagonist wherein the N-terminal amino acid residue of the PTH antagonist starts at any position spanning from position 8 through position 34 of SEQ ID NO:1 (PTH_{1-84}). The Applicant respectfully asserts that current claim 14 and its dependent claims are fully supported by the specification and claims as originally filed, for example, by virtue of the construction of original claim 14 that clearly, if not inherently, encompasses the currently claimed pharmaceutical PTH antagonist compositions.

Rejections under 35 U.S.C. § 112, Second Paragraph

Claims 14-38 are rejected under 35 U.S.C. § 112, second paragraph, as allegedly indefinite. The Office has specifically indicated that claim 14 is allegedly unclear whether "a pharmaceutical

PTH antagonist" is intended to mean the protein polypeptide or a pharmaceutical composition thereof. Claim 14 is amended herein to more clearly indicate the nature of the claimed pharmaceutical PTH antagonist compositions. Accordingly, withdrawal of this rejection is respectfully requested.

Claims 14, 18, 22, 26, 30 and 34 are allegedly unclear regarding what meaning of "conservatively substituted" is intended. In response, the Applicant asserts that one of skill in the art would understand the meaning of the term conservatively substituted variant of SEQ ID NO:1 (PTH_{1-84}). This genus thus defines a class which is designed to include variants of the disclosed proteins which might occur in individual subjects, but retain the useful properties of SEQ ID NO:1 as described. Applicant points out that there can be minor variations between individuals as to the precise amino acid sequence of any particular protein. Nevertheless, the Applicant has removed the disputed phrase with the understanding that they are entitled to a reasonable scope of equivalents of the present claims. Accordingly, withdrawal of this rejection as it applies to the amended claims is respectfully requested.

Claim 17 is cited as indefinite for the recitation of "further comprising a pharmaceutical carrier. The Office indicated that it is unclear how "a . . . antagonist" of claim 14 can comprise a carrier. As claim 17 is presently cancelled, this rejection is considered moot. Withdrawal of this rejection is respectfully requested.

Claims 18 and 30 are allegedly indefinite for the limitation "therapeutically effective" as it is purportedly unclear what purpose of this phrase was intended in light of the claim preamble. With further regard to claim 18, the Office has indicated that it is unclear whether an *in vivo* use is intended. As claim 18 is cancelled herein this rejection is rendered moot as it applies to this claim. Moreover, claim 30 is amended to clarify the intended meaning of the disputed phrase and to more particularly define the claim.

Claim 38 is allegedly indefinite due to the recitation of the phrase "a pulsatile manner" as the intended meaning is purportedly not defined in the specification. In response, the Applicant asserts that administration of a therapeutic peptide in a pulsatile manner is an art recognized method of treatment. One of skill in the art would understand the meaning of this phrase in the context of the present disclosure and claim 38. For example, in the treatment of osteoporosis, administering therapeutic in a pulsatile, intermittent or cyclic manner is known (*i.e.*, via an injection once a day, pulsatile transdermal iontophoretic administration, or other "pulsatile" methods, *see, e.g.*, S.

Lotinun, *et al.*, *Endocrine* 17(1):29-36 (2002); Y. Suzuki, *et al.*, *J. Pharm. Sci.* 91(2):350-61 (2002)). Accordingly, withdrawal of this rejection as it applies to claim 38 is respectfully requested.

Rejections under 35 U.S.C. § 112, First Paragraph

Claims 18-21 and 30-33 are rejected under 35 U.S.C. § 112, first paragraph, as allegedly not enabled. The Office has indicated that "while being enabling for claims limited in scope to a method for inhibiting the binding of PTH, or a method for in vivo decreasing calcium ion concentration in blood, does not reasonably providing enablement for claims to a method for *affecting* the binding of PTH (claims 18-21), or a method for in vivo *modulation* of calcium ion concentration in blood (claims 30-33)." April 30, 2003 Office Action, page 3. The Office has specifically indicated that the term "modulation" reads on both increasing and decreasing calcium ion concentration, and the term "affecting" reads on both stimulating and inhibiting binding. *Id.* The Office concludes by indicating that the claimed "methods would be enabled for inhibiting the binding of PTH [sic] to the receptor, and decreasing blood calcium concentration, they are not enabled for stimulating the binding, or increasing the blood calcium concentration." *Id.* at page 4.

The Applicant respectfully acknowledges the Office's indication that claims limited to a method for *in vivo* decreasing calcium ion concentration in blood are enabled by the present specification.

As claim 18 is cancelled herein this rejection is rendered moot as it applies to this claim. Although the Applicant respectfully asserts that the intended meaning of the terms "modulation" and "affecting" are clear based on the teachings provided in the specification, claim 30 is amended herein in accordance with the suggestions provided by the Office. Withdrawal of this rejection as it applies to claim 30 is respectfully requested.

Rejections under 35 U.S.C. § 102(b)

Claims 14-25 and 30-33 are rejected under 35 U.S.C. § 102(b), as allegedly anticipated by European Patent No. 0528271 A1 (Fukuda). The Office indicates that "Fukuda indicates that the prior art has established that compounds in which several amino acid residues on the N-terminal side of PTH (1-34) are deleted are known as inhibitors (page 3, lines 6-7)." Fukuda cites Horiuchi, et al., Science 220:1053-55 (1983), for this proposition. However, upon review of Horiuchi, it appears that the "inhibitor" compound in Horiuchi is a synthetic analog of bovine PTH comprising [tyrosine-34]bPTH-(7-34)NH_2. This is quite different than the claimed human PTH_{1-84} peptide antagonists

which lack one or more N-terminal amino acid residues. There does not appear to be any indication in Fukuda that PTH peptides missing one or more amino residues on the N-terminal side of PTH_{1-84}, while having an intact C-terminus, are known PTH antagonists. Incidentally, recent work has indicated that PTH peptide fragments such as PTH_{7-34} do not exhibit PTH antagonistic activity as the PTH antagonists having an intact C-terminus as currently described and claimed. *See* P. Divieti, *et al.*, *Endocrinology* 143(1):171-76 (2002) (attached hereto) (Divieti).

The Office has further indicated that "Fukuda discloses several hPTH muteins, SEQ ID Nos:19-22, which comprise the deletion of 3 to 6 amino acid residues on the N-terminal side of the sequence of hPTH, and teaches that these muteins function as antagonists of hPTH (page 3, lines 12-13)." April 30, 2003 Office Action, page 4.

In response, the Applicant respectfully directs the Office's attention to the present amendments to the pending claims and the discussion that follows. In particular, present composition claims 14 and 16 are limited to PTH antagonists having characteristics that lie outside of the teachings and suggestions of Fukuda.

Moreover, the Applicant respectfully asserts that Fukuda has failed to describe and enable the described "muteins" as PTH antagonists as such are described and claimed in the present application. For instance, it appears that the Fukuda description provides muteins that have the same "specific activity" or lower "specific activity" as compared with whole human PTH. See "Experimental Example" in the Specification at page 23. It appears that at least one of the "muteins" tested in this Example exhibited the same specific activity as whole human PTH. Id. For example, the table in this Example lists the specific activity of human PTH as 1.0, it further lists the specific activity of [Leu8]human PTH (a purported PTH mutein) as 1.0. As further indicated in the table, [Leu8]human PTH exhibits a specific activity of either 1.0 or 0.2, [Leu8, 18]human PTH exhibits a specific activity of 0.3, and [Cys35] human PTH exhibits a specific activity of 0.6. Each of these activities is the same or less than the activity of human PTH. In contrast, the present application describes and claims PTH antagonists that can be characterized as agonists with inverse biological activity to that exhibited by PTH.

Fukuda appears to be describing PTH muteins that operate through the normal PTH1/PTHrp receptor pathway but at a decreased rate to that of regular PTH, i.e., 0.2, 0.3, etc., the effect of PTH. This decreased rate may be due to saturation of the PTH receptors and down regulation of the PTH

effect. This mechanism lies in direct contrast to the PTH antagonists of the present claims, as the claimed antagonists would have no effect within the normal PTH1/PTHrp receptor pathway. Moreover, the claimed PTH antagonists stimulate a pathway having biological effects that lie in direct contrast to normal PTH pathway. For example, where PTH (and Fukuda's PTH muteins) would induce increase circulating calcium levels and induce hypercalcemia, the presently claimed PTH antagonists decrease circulating calcium levels and counter hypercalcemia. Thus, under the parameters provided in Fukuda, the presently claimed PTH antagonists would not register any PTH "specific activity" (as described at page 23 of Fukuda), while they separately induced a pathway having biological effects that lie in direct contrast to normal PTH pathway.

Moreover, it is unclear which, if any, of the tested "muteins" comprise PTH having only a selection of amino acids missing from the N-terminal portion of PTH_{1-84} deleted, without any additional modification to the whole PTH sequence. Importantly, a PTH antagonist, as described in the present description, does not have a lowered PTH activity level. Rather, the currently described PTH antagonists have an inhibitory effect on the normal PTH1/PTHrp receptor pathway. Based on results provided therein, Fukuda's "PTH antagonists" appear to merely have a lowered PTH activity level. There is no indication in Fukuda regarding how one of skill in the art would achieve an inhibitory effect on the normal PTH1/PTHrp receptor pathway utilizing the "muteins" described therein.

In addition, Fukuda appears to describe a very large set of PTH "muteins" that could be obtained by varying one or more of a variety of parameters. For example, the specification provides:

> [A] human parathyroid hormone mutein comprising at least one modification which is selected from the group consisting of (i) deletion of 3 to 6 amino acid residues on the N-terminal side in the amino acid sequence of human parathyroid hormones, (ii) substitution of another lipophilic amino acid residue for at least one methionine residue in said amino acid sequence, and (iii) substitution of a cysteine residue for one amino acid residue within the region of amino acid residue Nos. 34 to 47 in said amino acid sequence.

Page 3, lines 16-21. This reference fails to indicate which of the numerous potential candidate "PTH muteins" exhibit antagonistic PTH activity. As indicated above, the only presented

candidates fail to exhibit antagonistic activity as currently claimed. Importantly, Fukuda does not provide any evaluation of the "specific activity" of a PTH peptide lacking one or more N-terminal amino acids without further modification to the PTH sequence. Thus, Fukuda does not teach that PTH peptides lacking one or more N-terminal amino acids, as currently claimed, have inhibitory action on PTH.

Accordingly, Fukuda fails to teach PTH antagonists that exhibit PTH antagonistic activity as presently claimed in the method claims (*i.e.*, PTH antagonists that decrease calcium ion concentration in blood). Moreover, with regard to claims 14 and 16, Fukuda does not specifically teach or suggest a PTH antagonist having an N-terminal amino acid residue of the PTH antagonist starting at any position spanning from position 8 through position 34 of SEQ ID NO:1 (PTH_{1-84}); and the C-terminal amino acid residue of the PTH antagonist ending at position 84 of SEQ ID NO:1 (PTH_{1-84}).

As an anticipatory reference must teach each and every claim limitation, Fukuda does not anticipate the present claims. Withdrawal of this rejection is respectfully requested.

Rejections under 35 U.S.C. § 103(a)

Claims 26-29 and 34-38 are rejected under 35 U.S.C. § 103(a), as allegedly unpatenable over Fukuda in view of European Patent No. 0451867 (Kanmera). The Office has indicated that "Fukuda does not teach a method for treating renal osteodystrophy or osteoporosis with the hPTH antagonists." April 30, 2003 Office Action, page 5. The Office further provided that "Kanmera discloses peptides that are PTH antagonists, and teaches that the derivatives exhibit a potent inhibitory activity against hPTH and are useful as a therapeutic agent for treating dysbolism associated with calcium phosphoric acid, such as osteoporosis and renal osteodystrophy." *Id.* The Office argues that motivation to utilize Fukuda's "PTH antagonists" to treat disorders such as osteoporosis and renal osteodystrophy is provided by Kanmera, which purportedly teaches that "PTH antagonists are useful for such disorders." *Id.* at pages 5-6. Moreover the Office asserts that one of skill in the art would have expected success in the use of Fukuda's "PTH antagonists" in treating the disorders provided in Kanmera because Kanmera had purportedly "demonstrated that PTH antagonists are useful as a therapeutic agent." *Id.* at page 6. The Applicant respectfully traverses.

Kanmera describes parathyroid hormone related peptides (PTHrP) as peptides analogous to PTH "having an activity similar to PTH." Page 2, line 4. PTHrP apparently is a polypeptide comprised of 141 amino acids, rather than the 84 amino acids that comprise human PTH. Kanmera indicates that PTHrP's biological activity is "PTH-like," and has purportedly found a certain class of PTHrP's derivatives that have "excellent antagonistic activity against PTH." Interestingly, Kanmera references PTH_{3-34}, PTH_{7-34}, and their derivatives as "known" useful PTH antagonists, without a supporting citation. Page 2, line 11. Nevertheless, as indicated in the Divieti article (cited above and attached hereto), such PTH fragments do not exhibit the same antagonistic activity as those that are currently claimed (*see, e.g.*, Figures 1 and 5, and accompanying description in Divieti).

It appears to be the object of Kanmera to provide PTHrP peptides with increased antagonistic activity compared with "known" PTH peptide antagonists, and that these may be useful as therapeutic agents for certain disorders. Indeed, Kanmera provides that certain PTH derivatives lack sufficient activity for clinical use and that extensive investigation was undertaken to find PTH antagonists having sufficient antagonistic activity. Page 2, lines 15-17, 47-58. Incidentally, Kanmera seems to define "PTH antagonists" as compounds having an inhibitory effect on PTH action. Thus, regardless of the veracity of the assertion that PTH_{3-34}, PTH_{7-34}, and their derivatives are known PTH antagonists, Kanmera requires actual PTH antagonists (i.e., having an inhibitory effect on PTH action) as therapeutic agents. As indicated hereinbefore, Fukuda does not provide PTH antagonists within the common meaning of this term, and importantly, not within the meaning attributed to such compositions as provided in Kanmera.

Accordingly, as Kanmera contemplates the use of compositions that have an inhibitory effect on PTH action within its described methods, the compositions indicated in Fukuda (which lack such inhibitory activity) would not be expected to work in Kanmera with any expectation of success. Thus, one of skill in the art would not have been motivated to combine the teachings of Fukuda with Kanmera for the treatment of hypercalcemia, osteoporosis, hyperparathyroidism, renal osteodystrophy, and the like.

Moreover, Kanmera does not appear to fill the deficiencies of Fukuda indicated above that would render the above claims obvious. Thus, a combination of Fukuda with Kanmera would not result in the presently claimed subject matter. As such, withdrawal of this rejection is respectfully requested.

CONCLUSION

In view of the above, each of the presently pending claims in this application is believed to be in condition for allowance. Accordingly, the Examiner is respectfully requested to withdraw the outstanding rejection of the claims and to pass this application to issue. If it is determined that a telephone conversation would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number given below.

In the unlikely event that the transmittal letter is separated from this document and the Patent Office determines that an extension and/or other relief is required, applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to **Deposit Account No. 03-1952** referencing docket no. 532212000300. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Dated: July 30, 2003

Respectfully submitted,

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Human PTH-(7-84) Inhibits Bone Resorption *in Vitro* Via Actions Independent of the Type 1 PTH/PTHrP Receptor

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The linear sequence of intact mammalian PTH consists of 84 amino acids, of which only the most amino(N)-terminal portion, *i.e.* PTH-(1-34), is required for the classical actions of the hormone on mineral ion homeostasis mediated by the type 1 PTH/PTHrP receptor (PTH1R). Like the N-terminus, the carboxyl (C)-terminal sequence of PTH is highly conserved among species, and various circulating PTH C-fragments are generated by peripheral metabolism of intact PTH or are directly secreted, in a calcium-dependent manner, by the parathyroid glands. Certain synthetic PTH C-fragments exert actions on bone and cartilage cells that are not shared by PTH-(1-34), and specific binding of PTH C-peptides has been demonstrated in bone cells in which PTH1R expression was eliminated by gene targeting. The peptide human (h) PTH-(7-84) recently was shown to inhibit the calcemic actions of hPTH-(1-34) or hPTH-(1-84) in parathyroidectomized animals. To determine whether this anticalcemic effect of hPTH-(7-84) *in vivo* might result from direct actions on bone, we studied its effects on both resorption of intact bone *in vitro* and formation of osteoclasts in primary cultures of murine bone marrow. Human (h) PTH-(7-84) (300 nM) reduced basal 72-h release of preincorporated ^{45}Ca from neonatal mouse calvariae by 50% (9.6 ± 1.9% *vs.* 17.8 ± 5.7%; $P < 0.001$) and similarly inhibited resorption induced by hPTH-(1-84), hPTH-(1-34), 1,25-dihydroxyvitamin D_3 (VitD), PGE_2, or IL-11. In 12-d murine marrow cultures, both hPTH-(7-84) (300 nM) and hPTH-(39-84) (3000 nM) lowered VitD-dependent formation of osteoclast-like cells by 70%. On the contrary, these actions of hPTH-(7-84) were not observed with the PTH1R antagonists hPTH-(3-34)NH_2 and [L^{11},D-W^{12},W^{23},Y^{36}]hPTHrP-(7-36)NH_2, which, unlike hPTH-(7-84), did inhibit PTH1R-dependent cAMP accumulation in ROS 17/2.8 cells. We conclude that hPTH-(7-84), acting via receptors distinct from the PTH1R and presumably specific for PTH C-fragments, exerts a direct antiresorptive effect on bone that may be partly due to impaired osteoclast differentiation. (*Endocrinology* 143: 171–176, 2002)

INTACT PTH from different mammalian species comprises 84 amino acids, the sequence of which is highly conserved within both its amino (N)-terminal and carboxyl (C)-terminal regions (1). The first 34 amino acids of PTH, including an intact N-terminus, are both necessary and sufficient for the classical actions of the hormone on mineral ion homeostasis and bone metabolism. These effects of intact and N-terminal PTH are mediated through the type 1 PTH/PTH-related peptide receptor (PTH1R), a G protein-coupled receptor that can activate both adenylate cyclase and PLC (2).

The parathyroid glands are the main source of PTH, although small amounts of its mRNA were recently identified in hypothalamus and spleen (3). PTH synthesis and secretion are tightly controlled by calcium via a membrane-bound calcium-sensing receptor (4), although vitamin D (5, 6) and phosphate (7, 8) also play modulating roles. Under physiological conditions, a portion of the newly synthesized hormone undergoes intraglandular cleavage at a rate that also is regulated by extracellular calcium (9, 10). This cleavage results in the cosecretion of intact PTH and various C-terminal fragments, the predominant forms of which, identified to date, consist of peptides with N-termini located between residues 24 and 43 (11–13). Secreted intact PTH also undergoes endopeptidic cleavage(s) in peripheral tissues, mainly liver and kidney, by processes that degrade the resulting N-terminal fragments *in situ* but release additional C-fragments into the circulation (14–16). As a consequence of their obligatory renal clearance, the concentration of circulating C-terminal PTH (CPTH) fragments increases dramatically in patients with renal failure (17–19).

Recently, fragments of PTH lacking residues at the extreme N-terminus but otherwise large enough to cross-react with most commercially available intact PTH two-site immunoassays were detected after HPLC fractionation of normal plasma and, at much higher levels, in plasma of patients with advanced renal failure (19). Although their precise structure(s) has not been ascertained, these fragments exhibit chromatographic properties similar to those of synthetic PTH-(7-84) (18). Interestingly, human (h) PTH-(7-84) was recently shown to inhibit the calcemic actions of PTH-(1-84) and PTH-(1-34) in parathyroidectomized animals at doses much lower than would be predicted to effectively antagonize either hormonal form at the PTH1R (20, 21). Thus, these *in vivo* observations suggest that CPTH fragments might act upon bone cells via one or more mechanisms independent of the PTH1R *per se*.

The possibility that CPTH fragments (as well as intact PTH) might activate receptors distinct from the PTH1R was first postulated over 2 decades ago when Arber *et al.* (22) showed that a particular CPTH fragment, PTH-(53-84), possessed biological properties different from those of PTH-(1–34). Subsequent work from several different groups has produced direct evidence that CPTH fragments from within the sequence PTH-(35-84) bind specifically to bone and kidney cells and/or membranes and can exert direct actions on target cells in bone or cartilage. For example, CPTH frag-

Abbreviations: CPTH, C-terminal PTH; hPTH, human PTH; PTH1R, type 1 PTH/PTHrP receptor; TRAP, tartrate-resistant acid phosphatase; TRAP+MNC, TRAP-positive cells containing three or more nuclei (osteoclast-like multinucleated cells); VitD, 1,25-dihydroxyvitamin D_3.

ments such as hPTH-(53-84) and hPTH-(60-84) increased alkaline phosphatase activity and expression of mRNAs for both alkaline phosphatase and osteocalcin in bone-derived cells and induced transient increases in cytosolic free calcium in chondrocytes (23–26). Photoaffinity cross-linking studies to characterize the receptors for CPTH fragments (*i.e.* CPTHRs) expressed by ROS 17/2.8 osteosarcoma and rPT parathyroid cells were performed by Inomata *et al.* (27) using radioiodinated (Leu^{8,18},Tyr^{34})hPTH-(1-84) and (Tyr^{34})hPTH-(19-84), neither of which binds well, if at all, to the PTH1R. These studies showed that in ROS 17/2.8 cells, two proteins (80 and 30 kDa) interacted specifically with the radioligands used, whereas in rPT cells, only the 80-kDa protein was observed. Affinity labeling was inhibited by hPTH-(1-84), hPTH-(19-84), and, to a lesser extent, by CPTH fragments that were truncated even further at the N-terminus, whereas hPTH-(1-34) had no effect (27). Recently, hPTH-(7-84) was shown to bind to CPTHRs on ROS 17/2.8 cells with affinity comparable to that of hPTH-(1-84) (21).

Unequivocal evidence that such CPTHRs are distinct from the PTH1R was provided by our recent demonstration that specific [^{125}I](Tyr^{34})hPTH-(19-84) binding is observed in clonal osteoblastic and osteocytic cell lines derived from mice in which the PTH1R gene had been eliminated by gene targeting (28). Further, CPTH fragments such as hPTH-(39-84) were shown to regulate cellular functions (*i.e.* connexin 43 expression and apoptosis) in clonal PTH1R-null osteocytes at concentrations shown to bind effectively to CPTHRs in these cells (28).

Thus, the expression of CPTHRs in bone offers a plausible mechanism by which circulating PTH fragments, truncated at their N-termini and including peptides as long as hPTH-(7-84) might exert biological actions, potentially different from those of intact PTH, by a means other than direct antagonism at the PTH1R. To determine whether the ability of hPTH-(7-84) to antagonize the calcemic response to PTH-(1-84) *in vivo* might reflect direct actions of this C-PTH fragment on bone, we studied its effects using *in vitro* assays of osteoclast formation and bone resorption.

Materials and Methods

Materials

Culture media were obtained from the Media Kitchen (Pediatric Surgery, Massachusetts General Hospital, Boston, MA), other tissue culture reagents were purchased from Life Technologies, Inc. (Grand Island, NY), and additional reagents and chemicals were obtained from Sigma (St. Louis, MO) or Fisher (Pittsburgh, PA). Recombinant hPTH-(1-84) was a gift from Chugai Pharmaceutical Co. (Shizuoka, Japan), and hPTH-(7-84) and [D^{76}]hPTH-(39-84) were purchased from Bachem (Torrance, CA). All other PTH fragments, including the PTH1R antagonist (Leu^{11},D-Trp^{12},Trp^{23},Tyr^{36})hPTHrP-(7-36)amide PTHrP-(7-36) (29, 30) and hPTH-(3-34)-amide, were synthesized at Massachusetts General Hospital Peptide and Oligonucleotide Core Laboratory (Boston, MA). Recombinant mouse IL-11 was purchased from R&D Systems, Inc. (Minneapolis, MN), and VitD was obtained from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA).

Animals

Animals were maintained in facilities operated by the Massachusetts General Hospital Center for Comparative Resources in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were employed using protocols approved by the institutional animal care and use committee.

Bone resorption assay

Bone resorption was quantitated by the release of previously incorporated ^{45}Ca from newborn mouse calvarial bones *in vitro* (31). Briefly, calvaria from 3- to 4-d-old mice (CD-1 strain, Charles River Laboratories, Inc., Wilmington, MA) were obtained after maternal administration of 50 μCi $^{45}CaCl_2$ (NEN Life Science Products, Boston, MA), sc, on the 19th day of gestation. The bones were divided in half and precultured in 1 ml DMEM containing 1 mM calcium, 2 mM phosphate, 5% heat-inactivated horse serum, and 1% antibiotic/antimycotic solution (Life Technologies, Inc.) on a rocking platform at 90 oscillations/min in a 37 C incubator under 5% CO_2 in air. After 24 h the medium was replaced with 1 ml fresh medium containing the test substances (or vehicle alone). After an additional 72 h, the bones were removed, rinsed three times in PBS, placed in scintillation vials containing 0.4 ml 2 N HCl, and incubated for 2 h at room temperature before addition of 5 ml scintillation fluid (Packard Instruments, Downers Grove, IL). Aliquots of culture medium (0.5 ml) were transferred to separate vials containing 5 ml scintillation fluid for determination of released radioactivity. In some experiments additional aliquots of culture medium were used for measurements of cAMP as described below. Bone resorption was determined as the percentage of total initial bone ^{45}Ca subsequently released into the medium during the 72-h treatment period. Results are expressed as the mean ± SEM of the percentage of ^{45}Ca released for groups of four bones and are representative of at least three independent experiments.

Bone marrow culture

Bone marrow cells were isolated as previously described (32). Briefly, 4- to 6-wk-old male mice (C57B/6 strain, Charles River Laboratories, Inc.) were killed by carbon dioxide asphyxiation, and tibias and femurs were aseptically removed and dissected free of adhering tissue. The metaphyses were removed, and the marrow cavity was flushed with 1 ml αMEM to obtain marrow cells, which were collected into 50-ml tubes and washed twice with αMEM. Cells were cultured in growth medium [αMEM containing 10% FBS (lot 1011961 Life Technologies, Inc.) and 1% penicillin-streptomycin] containing 100 nM dexamethasone (Sigma) after plating at 1.5 × 10^6 cells/well in 24-well plates. Half of the culture medium was replaced 3 times/wk with fresh medium containing a 2× concentration of the test substances (or vehicle). All cultures were maintained in a 37 C incubator under 5% CO_2 in air. After culture for 10 d, cells adherent to the surface of each well were rinsed twice with PBS, fixed with 10% formalin in PBS for 10 min at room temperature and with ethanol/acetone (50:50, vol/vol) for 1 min before staining for tartrate-resistant acid phosphatase (TRAP), as previously described (33). TRAP-positive cells containing 3 or more nuclei were scored as osteoclast-like multinucleated cells (TRAP+MNCs). Cells were counted at ×10 magnification in 20 contiguous fields along 2 orthogonal pathways in each well, a method previously employed to account for the nonuniform distribution of cells within wells (33). The number of TRAP+MNCs contained in these 20 fields was expressed as the number per well.

cAMP accumulation

Clonal rat osteosarcoma cell (ROS 17/2.8) were cultured in 48-well plates in Ham's F-12 medium (Life Technologies, Inc.) supplemented with 10% FBS and 1% penicillin-streptomycin. The cultures were maintained for 5–7 d after reaching confluence by replacing the medium every other day. To assess basal and agonist-induced cAMP accumulation, cells were rinsed twice with assay buffer (DMEM containing 2 mM isobutylmethylxanthine, 1 mg/ml heat-inactivated BSA, and 35 mM HEPES-NaOH, pH 7.4) and then incubated for 45 min at 23 C with the same buffer alone or in the presence of different peptides (or with conditioned medium collected from resorption assays). The buffer then was rapidly aspirated, the plates were frozen on powdered dry ice, and the frozen cells were subsequently thawed directly into 0.25 ml 50 mM HCl. Cell-associated cAMP in the acid extracts was measured as previously described (34). Results were expressed as picomoles of cAMP produced per well over 45 min.

Statistical analysis

Results are expressed as the mean ± SEM or the mean ± SD. The significance of differences between treatment and control groups was

assessed by the Mann-Whitney test. Data were analyzed using the PRISM 3.0 software package for Macintosh (GraphPad Software, Inc., San Diego, CA).

Results

The limited efficacy of short, amino-terminally truncated PTH or PTHrP analogs, such as hPTH-(3-34) or hPTHrP-(7-36), to antagonize the action of PTH-(7-34) or PTH-(1-84) *in vivo* or *in vitro* contrasts with the efficiency with which hPTH-(7-84) inhibits the calcemic action of hPTH-(1-84) [or hPTH-(7-34)] *in vivo* (20, 21). Although pharmacokinetic differences *in vivo* between N-truncated PTH fragments of different length and structure might explain these differences, we considered the alternative possibility that, unlike short N-truncated PTH or PTHrP fragments, this effect of hPTH-(7-84) *in vivo* might not be mediated via antagonism at the PTH1R. We therefore directly compared the effects of hPTH-(7-84) with those of PTHrP-(7-36) or hPTH-(3-34) in an *in vitro* assay of bone resorption that relies upon the release of pre-incorporated ^{45}Ca from neonatal murine calvarial bones. First, as shown in Fig. 1, addition of hPTH-(7-84) (300 nM) alone reduced basal ^{45}Ca release by approximately 50% [control, 17.8 ± 5.7%; hPTH-(7-84), 9.6 ± 1.9%; $P < 0.001$]. This effect was comparable to that of salmon calcitonin (100 nM; 9.9 ± 1.1%; $P < 0.001$). In contrast, no inhibition of basal resorption was observed with equimolar concentrations of much shorter N-truncated PTH analogs, such as hPTH-(3–34) (300 nM; 18.7 ± 4.2%) or PTHrP-(7-36) (300 nM; 15.4 ± 4.9%), that bind as well or more effectively to the PTH1R as hPTH-(7-84).

Further, as shown in Fig. 2, hPTH-(7-84) (300 nM) also significantly inhibited (by 50% or more) agonist-induced bone resorption caused by a variety of osteotropic agents, including intact hPTH-(1-84) (3 nM), hPTH-(1-34) (3 nM), VitD (10 nM), PGE_2 (100 nM), and IL-11 (10 ng/ml). The antiresorptive effect of hPTH-(7-84) was dose dependent, with an IC_{50} of approximately 200 nM (data not shown). We considered the possibility that these inhibitory actions of hPTH-(7-84) might reflect antagonism, at the PTH1R, of an effect of locally secreted PTHrP to augment the responses to these other agonists. As shown in Fig. 3, however, neither hPTH-(3-34) (1 μM) nor PTHrP-(7-36) (1 μM), both of which act as PTH1R antagonists (see below) at the concentrations used, inhibited resorption induced by VitD (10 nM). With respect to a possible nonspecific or irreversible toxic effect of hPTH-(7-84), we observed that removal of the peptide after 24 h of exposure to calvariae did not impair the

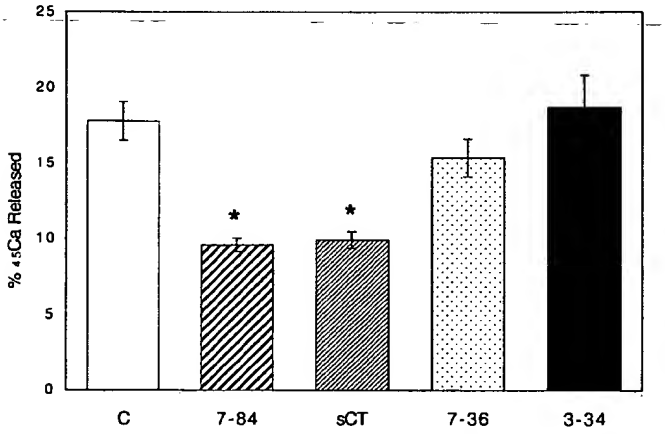

FIG. 1. Inhibition of basal bone resorption by hPTH-(7-84). Calvariae prelabeled with ^{45}Ca by maternal injection were isolated from neonatal mice as described in *Materials and Methods*. Bones were incubated individually, in treatment groups of four bones each, for 72 h after a single addition of vehicle alone (controls, C); hPTH-(7-84), hPTHrP-(7-36), or hPTH-(3-34) (all at 300 nM); or salmon calcitonin (sCT; 100 nM). Results are expressed as percentages of total ^{45}Ca released over 72 h. Values shown are the mean ± SEM of results from several (three to six) independent experiments. *, $P < 0.001$ *vs.* controls.

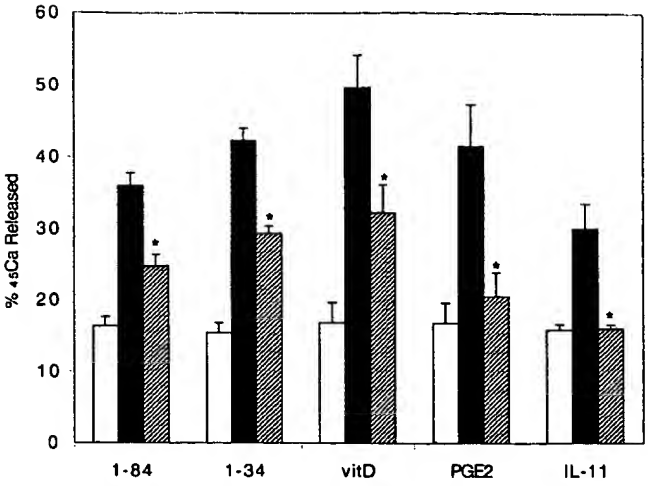

FIG. 2. Inhibition by hPTH-(7-84) of bone resorption induced by various osteotropic agents. Resorption assays were conducted as described in Fig. 1 for controls (□), osteotropic agents alone (■), or osteotropic agents in combination with 300 nM hPTH-(7-84) (▨). Osteotropic agents were employed at the following concentrations: hPTH-(1-84), 3 nM; hPTH-(1-34), 3 nM; VitD, 10 nM; PGE_2, 100 nM; and IL-11, 10 ng/ml. Results are expressed as the mean ± SEM of quadruplicate determinations. Each experiment was repeated three times. *, $P < 0.05$ for difference between osteotropic agent alone *vs.* osteotropic agent plus hPTH-(7-84).

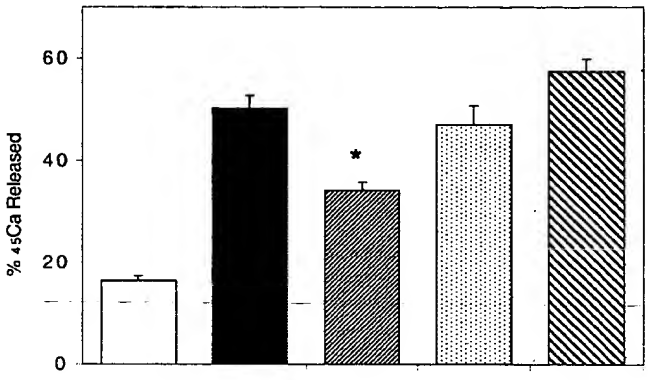

FIG. 3. Inhibition of VitD-induced bone resorption by hPTH-(7-84), but not by hPTHrP-(7-36) or hPTH-(3-34). Calvarial bones, incubated as described in Fig. 1, were treated with vehicle alone (C) or with 10 nM VitD, either alone (D) or together with 300 nM hPTH-(7-84) (D + 7-84), 1 μM hPTHrP-(7-36) (D + 7-36), or 1 μM hPTH-(3-34) (D + 3-34). Results are expressed as the mean ± SEM of quadruplicate determinations and are representative of three independent experiments. *, $P < 0.05$ between VitD alone and VitD plus PTH-(7-84).

resorptive response of the bones to subsequently added VitD (data not shown).

hPTH-(7-84) does not activate adenylyl cyclase or measurably inhibit the binding of a radiolabeled hPTH-(1-34) analog to PTH1Rs expressed on ROS 17/2.8 rat osteosarcoma cells, which also express CPTHRs (21, 27). To directly address the possibility that hPTH-(7-84) nevertheless might antagonize signaling by PTH1R agonists, either directly at the PTH1R or via activation of CPTHRs, ROS 17/2.8 cells were incubated with hPTH-(1-34) at a submaximal concentration (3 nM) in the absence or presence of excess hPTH-(7-84) (0.1–1 μM). As shown in Fig. 4, we observed no inhibition of the cAMP response to PTH-(1-34) by hPTH-(7-84) (0.1–1 μM), whereas both PTHrP-(7-36) and PTH-(3-34), when present at 1 μM, inhibited the response by 50%. In related experiments undiluted samples of conditioned medium from calvarial resorption assays (described above) to which 300 nM hPTH-(7-84), PTHrP-(7-36) or PTH-(3-34) had been added 72 h earlier also were tested for inhibition of hPTH-(1-34)-induced cAMP accumulation in ROS 17/2.8 cells. In none of these samples was inhibition of the cAMP response to hPTH-(1-34) observed (data not shown).

Inhibition of bone resorption by hPTH-(7-84) could result from decreased osteoclast formation, inhibition of the activity or survival of mature osteoclasts, or both. To determine whether hPTH-(7-84) impairs osteoclast formation, the activity of this fragment was studied in cultures of whole murine bone marrow. As shown in Fig. 5A, hPTH-(7-84) alone exerted no effect on the formation of TRAP+MNCs, although basal osteoclast formation in this system is low, and an inhibitory effect therefore might not be easily detectable. On the other hand, when osteoclast formation was stimulated by VitD (10 nM), hPTH-(7-84) (300 nM) caused a striking (70%) reduction in the formation of TRAP+MNCs relative to the effect of VitD alone [VitD, 153 ± 38 cells; VitD + hPTH-(7-84), 53 ± 14 cells]. In contrast,

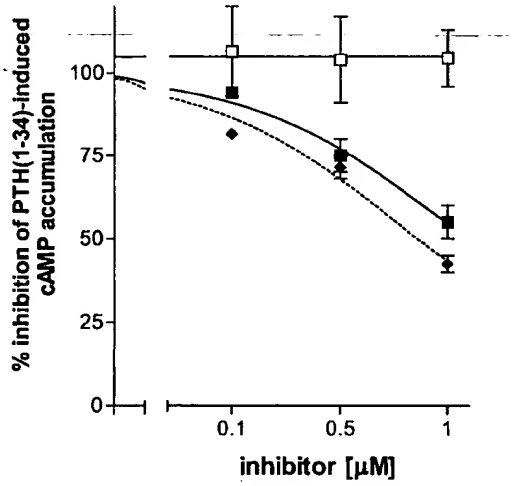

FIG. 4. Inhibition of cAMP accumulation in PTH-treated ROS 17/2.8 cells. Cells were stimulated with an approximately half-maximal concentration of PTH-(1-34) (3 nM) in the absence or presence of increasing concentrations of PTH-(7-84) (□, *solid line*), PTH-(3-34) (◆, *dashed line*), or PTHrP-(7-36) (■, *solid line*). Data are expressed as percentages of the cAMP response to 3 nM PTH-(1-34) alone and represent the results (mean ± SEM) of at least two independent experiments.

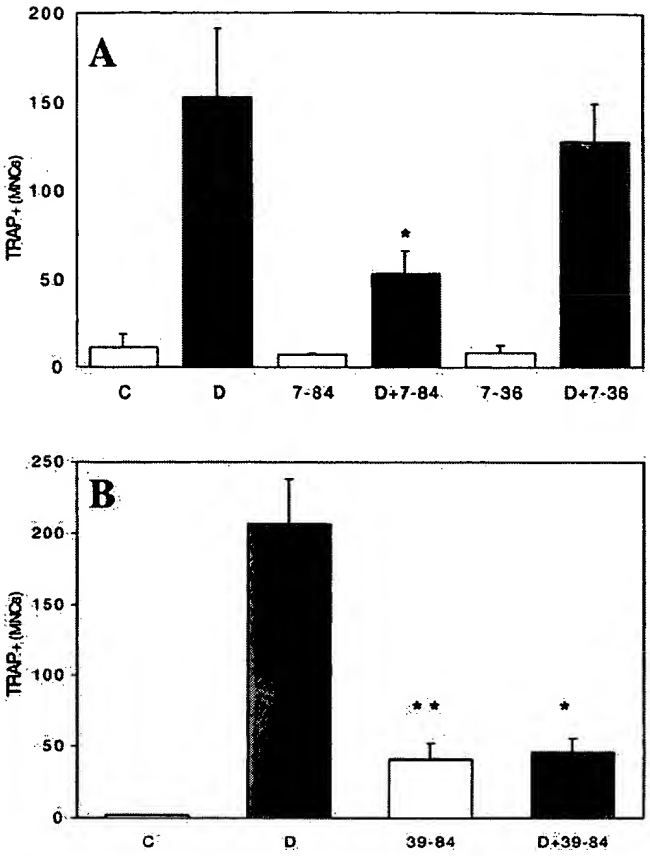

FIG. 5. Inhibition of osteoclast generation by hPTH-(7-84) and hPTH-(39-84). Whole bone marrow was isolated and cultured as described in *Materials and Methods*. Adherent and nonadherent cells were maintained in culture for 12 d, and the indicated treatments were added three times weekly, as described in *Materials and Methods*. At the end of the culture period, cells were fixed and stained for TRAP. A: C, Control; D, VitD (10 nM); 7-84, hPTH-(7-84) (300 nM); 7-36, hPTHrP-(7-36) (300 nM). B: C, Control; D, VitD (10 nM); 39-84, hPTH-(39-84) (3000 nM). Values (number of cells per well) are expressed as the mean ± SD for triplicate determinations (see *Materials and Methods*). Experiments were repeated twice. *, $P < 0.05$ *vs.* VitD alone; **, $P < 0.05$ *vs.* control.

PTHrP-(7-36) did not inhibit TRAP+MNC formation induced by VitD [VitD + PTHrP-(7-36), 127 ± 22 cells; Fig. 5A]. To determine whether shorter C-terminal PTH fragments could regulate osteoclast formation, we tested the effects of hPTH-(39-84), alone or in combination with VitD (10 nM). As shown in Fig. 5B, hPTH-(39-84) alone, at 3000 nM, slightly stimulated osteoclast formation (41 ± 11 cells), as reported previously for short CPTH fragments (24, 33). Like hPTH-(7-84), however, hPTH-(39-84) dramatically inhibited osteoclast formation promoted by VitD [VitD, 207 ± 31 cells; VitD + hPTH-(39-84), 46 ± 10 cells].

Discussion

Recent studies demonstrating that the extended CPTH fragment hPTH-(7-84) can inhibit the calcemic effects of PTH-(1-84) and PTH-(1-34) in thyroparathyroidectomized animals have suggested that CPTH peptides, normally present in blood and previously assumed to be biologically inert prod-

ucts of PTH metabolism, may be physiologically active (20, 21). As the hypocalcemic actions of hPTH-(7-84) described *in vivo* were associated with lowering of serum phosphate but were not accompanied by significant changes in urinary calcium or phosphate excretion (20, 21), a primary effect of this CPTH peptide on bone seems likely. The present *in vitro* studies were directed at clarifying whether hPTH-(7-84) might act directly on bone to inhibit the action of hPTH-(1-84) or hPTH-(1-34).

One possible mechanism for such an effect could involve direct antagonism by hPTH-(7-84) to prevent binding of hPTH-(1-34) or hPTH-(1-84) to PTH1Rs expressed on osteoblasts or marrow stromal cells. Because the antagonism *in vivo* is observed at much lower doses of hPTH-(7-84), relative to intact PTH, than that predicted to be necessary for direct antagonism at the PTH1R, however, we also considered the alternative possibility that hPTH-(7-84) may exert unique PTH1R-independent antiresorptive effects by activating CPTHRs expressed in bone cells. Our results are fully consistent with this latter hypothesis. Thus, we observed concentration-dependent inhibition of bone resorption in *ex vivo* calvarial organ cultures that was not mimicked by shorter, N-truncated PTH fragments that 1) are more effective PTH1R antagonists than is hPTH-(7-84) and 2) do not bind detectably to CPTHRs expressed on bone cells (28). Similar results were obtained in studies of osteoclastogenesis using whole bone marrow cultures, which further suggests that CPTHRs may be involved in the regulation of osteoclast formation. Because the number of mononuclear TRAP-positive cells formed in the marrow cultures also was reduced by hPTH-(7-84), the predominant action in osteoclastogenesis may be to inhibit formation of osteoclast precursors. The rapidity (1–2 h) of the hPTH-(7-84) effect observed *in vivo*, however, suggests that interference with the activity of mature osteoclasts also may be involved.

The antiresorptive effect of hPTH-(7-84) observed in the calvarial assay system contrasted sharply with the inability of hPTH-(3-34) or PTHrP-(7-36), introduced at similar concentrations, to inhibit resorption. Because both of these shorter, N-truncated peptides are effective *in vitro* PTH1R antagonists, whereas hPTH-(7-84) is not (as shown in Fig. 4), these results argue strongly against a mechanism involving direct antagonism by hPTH-(7-84) at the PTH1R of either endogenous PTHrP present within the cultured bones or exogenously added PTH. Moreover, the antiresorptive effect of hPTH-(7-84) *in vitro* was not restricted to resorption induced by added PTH, but was encountered in both control cultures and cultures treated with a variety of unrelated bone-resorbing agonists, including VitD, PGE_2 and IL-11. These findings point to a more generalized antiresorptive mechanism by which PTH-(7-84), presumably acting via CPTHRs, may limit the formation and, possibly, the activity of mature osteoclasts. This could reflect interference with the up-regulation of RANKL or macrophage colony-stimulating factor, the down-regulation of OPG, or both, that normally are triggered in marrow stromal cells and osteoblasts by these diverse resorbing agents (35). In this regard, we observed expression of CPTHRs by PTH1R-null osteoblasts and osteocytes (28) and by clonal marrow stromal cells that are capable of supporting PTH- or vitamin D-dependent osteoclast formation from hemopoietic progenitors *in vitro* (our unpublished observations). We also cannot yet exclude that the inhibition of resorption was mediated partly by a proapoptotic effect of hPTH-(7-84) on bone cells via activation of the CPTHR, as we previously reported in osteocytic cells (28). Moreover hPTH-(7-84) could act directly on mature osteoclasts, their hemopoietic precursors, or both to dampen cellular responsiveness to activation of RANK or c-*Fms* by their respective stromal cell or osteoblast-derived ligands. Indeed, evidence that shorter CPTH fragments alone can modestly induce osteoclast formation, as seen in the present study with hPTH-(39-84) and reported previously (24, 33), in contrast to the inhibitory effects of the same fragment upon osteoclast formation induced by vitamin D, points to a complexity in CPTHR action that is not readily explained at present, but that could involve disparate effects on distinct cell types involved in osteoclast formation. Direct analysis of CPTHR expression in such cells would be needed to address this possibility.

One prediction of our results might be that PTH-(1-84), which binds to CPTHRs with affinity comparable to that of PTH-(7-84) (21), should elicit less bone resorption than PTH-(1-34), which does not interact effectively with CPTHRs (28). Although few direct *in vitro* comparisons have been performed (24, 36), the available data do not indicate substantial or consistent differences in resorptive responses to these two peptides. Indeed, we observed in the calvarial resorption assay that the intact hormone reproducibly induced less ^{45}Ca release than did PTH-(1-34) at equimolar concentrations, although this difference was never statistically significant. This could indicate that when PTH1Rs and CPTHRs are exposed simultaneously to equimolar concentrations of a common ligand, the PTH1R-mediated resorptive response strongly predominates. Alternatively, it is possible that despite comparable binding affinity, intact PTH cannot activate CPTHRs as effectively as N-truncated peptides (by analogy with the disparate activation of PTH1Rs observed with PTH-(1-34) *vs.* PTH-(3-34)). On the other hand, because CPTH fragments normally circulate in plasma at concentrations at least 5- to 10-fold higher than those of intact PTH, a requirement for higher molar concentrations of CPTH ligands to activate CPTHRs might be expected. This concept is consistent with our finding that a 10- to 100-fold molar excess of CPTH ligand is needed to elicit functional antagonism of PTH1R-mediated resorption *in vitro*. The observation that hPTH-(7-84) could antagonize the calcemic response to hPTH-(1-84) at equimolar doses *in vivo* might be related to differential bioavailability or metabolism of the two peptides after their ip or iv administration (20, 21).

Secretion of CPTH fragments by the parathyroid glands is positively regulated by blood calcium (37). Thus, one possible physiological role of the antiresorptive action of N-truncated PTH fragments *in vivo* could be to modulate the extent of bone resorption induced by intact PTH in a manner responsive to the extracellular calcium concentration. Such a mechanism, for example, might allow for maximal release of calcium from bone only during severe hypocalcemia to supplement the ongoing renal and (indirect) intestinal actions of PTH. It is important to note that the chemical identities of all circulating CPTH fragments have not yet been completely defined. In particular, the existence in blood of PTH-(7-84) *per se* has not been directly demonstrated. On the other hand, the

recent immunochemical characterization of nonintact PTH peptides, which are especially abundant in renal failure, is consistent with the presence of extended CPTH fragments longer than those previously inferred from analyses of secreted or peripherally generated cleavage products, the N-termini of which ranged between positions 24 and 43 of the intact PTH sequence (19, 38). Thus, the possibility that PTH fragments similar or identical to PTH-(7-84) may be present in blood, especially in renal failure, at concentrations high enough to activate CPTHRs and thereby exert direct effects on bone resorption must be considered.

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